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Temporal control of gene expression in transgenic mice by a tetracycline-responsive promoter

PRISCILLA A. FURTH†‡, LUC ST. ONGE†, HINRICH BÖGER†, PETER GRUSS†, MANFRED GOSSEN§, ANDREAS KISTNER§, HERMANN BUJARD§, AND LOTHAR HENNIGHAUSEN†¶‖

†Department of Molecular Cell Biology, Max Planck Institute for Biophysical Chemistry, Am Fassberg, 37018 Göttingen, Germany; ‡Division of Infectious Diseases, Department of Medicine, University of Maryland Medical School, Baltimore, MD 21201; §Zentrum für Molekulare Biologie, Universität Heidelberg, 69120 Heidelberg, Germany; and ¶Laboratory of Biochemistry and Metabolism, National Institute of Diabetes, Digestive, and Kidney Diseases, National Institutes of Health, Bethesda, MD 20982

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ABSTRACT Promoters whose temporal activity can be directly manipulated in transgenic animals provide a tool for the study of gene functions *in vivo*. We have evaluated a tetracycline-responsive binary system for its ability to temporally control gene expression in transgenic mice. In this system, a tetracycline-controlled trans-activator protein (tTA), composed of the repressor of the tetracycline-resistance operon (*tet* from *Escherichia coli* transposon Tn*10*) and the activating domain of viral protein VP16 of herpes simplex virus, induces transcription from a minimal promoter ($P_{hCMV*-1}$; see below) fused to seven *tet* operator sequences in the absence of tetracycline but not in its presence. Transgenic mice were generated that carried either a luciferase or a β-galactosidase reporter gene under the control of $P_{hCMV*-1}$ or a transgene containing the tTA coding sequence under the control of the human cytomegalovirus immediate early gene 1 (hCMV *IE1*) promoter/enhancer. Whereas little luciferase or β-galactosidase activity was observed in tissues of mice carrying only the reporter genes, the presence of tTA in double-transgenic mice induced expression of the reporter genes up to several thousand-fold. This induction was abrogated to basal levels upon administration of tetracycline. These findings can be used, for example, to design dominant gain-of-function experiments in which temporal control of transgene expression is required.

Functions of mammalian gene products in development and oncogenesis have been defined by their actions in dominant gain-of-function experiments in transgenic animals (1). The transgenes in these experiments are controlled by either tissue-specific or ubiquitously expressed promoters. Their temporal and spatial expression patterns are dependent upon the characteristics of the promoters employed. However, numerous questions require control over timing and tissue-specific expression of a transgene.

Although several inducible systems have been established in transgenic mice, all have limitations. In the classic binary systems, the target gene is silent and can be activated upon crossing in a transgene that encodes either a trans-activator or a recombinase (2–4). In such systems, the temporal activity of the target gene is dependent on the expression pattern of the effector molecule (trans-activator or recombinase), and it cannot be directly regulated by changing experimental conditions. In systems based on environmental signals such as steroid hormones or heavy metal ions, gene expression can be modulated (for review, see ref. 5). However, generalized physiologic or toxic effects from the inducing chemicals and high basal-transcriptional activity from the promoters limit their utility. Finally, tissue-specific and hormone-inducible promoters, such as the long terminal repeat of mouse mammary tumor virus or the whey acidic protein gene promoter, direct gene expression to only a few selected tissues, and the timing of gene expression is primarily controlled by endogenous hormone levels (6, 7).

Yet another approach to control gene expression has been to adopt well-characterized regulatory systems from *Escherichia coli* for use in mammalian cells (8). Transgenic systems based on the *lac* operon have proven inadequate because of inefficient induction levels and kinetics (5). However, the development of a regulatory circuit based on the tetracycline-resistance operon *tet* from *E. coli* transposon Tn*10* opened a new approach to controlling transgene expression (9, 10). In this system, a fusion tetracycline-controlled trans-activator protein (tTA) composed of the *tet* repressor and the activating domain of viral protein VP16 of herpes simplex virus strongly activates transcription from $P_{hCMV*-1}$, a minimal promoter from human cytomegalovirus (hCMV) fused to *tet* operator sequences. The tTA binds to the *tet* operator sequences in the absence of tetracycline but not in its presence. This results in repression of transcription upon introduction of tetracycline. In the animal, tetracycline derivatives are readily absorbed and broadly distributed to different tissues with minimal toxicity at the concentration needed to regulate the activity of the synthetic promoter (11).

In this study we have evaluated the tetracycline-responsive regulatory system as a means to temporally regulate transgene expression in animals. The luciferase reporter gene was used as a sensitive measure of expression levels in whole tissues, and the β-galactosidase gene was used to monitor expression at the single cell level *in situ*.

MATERIALS AND METHODS

Generation of the Transgenes and Transgenic Mice. The tTA-encoding sequence contained in plasmid pUHG15-1 (P.G. and H.B., unpublished data) is under the control of the hCMV *IE1* promoter/enhancer ("hCMV-tTA gene") and is flanked at the 3′ end by the rabbit β-globin intron and a poly(A) signal. Plasmid pUHC13-3 containing the luciferase gene has been described (9). The β-galactosidase reporter gene containing a nuclear localization signal was constructed as follows: an *Xba* I–*Bgl* II fragment, containing the nuclear β-galactosidase structural gene, an intron, and a poly(A) signal, was excised from plasmid pNlacF (12) and cloned into a plasmid containing $P_{hCMV*-1}$. The β-galactosidase-encoding transcription unit was separated from the vector with *Xho* I and *Bgl* II and isolated as a 4.3-kb fragment from an agarose gel by using electroelution. The hCMV-tTA gene was isolated

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Abbreviations: rlu, relative light unit(s); tTA, tetracycline-controlled trans-activator; hCMV, human cytomegalovirus.

‖To whom reprint requests should be addressed; electronic mail lotharh@amb.niddk.nih.gov.

as a 2.7-kb *Xho* I–*Pfl*mI fragment, and the luciferase reporter gene was isolated as a 3.1-kb *Xho* I–*Eae* I fragment.

The DNA fragments were injected into fertilized eggs at a concentration of ≈5 ng per μl. Transgenic mice were generated according to standard procedures, and founder mice were analyzed by using th PCR and Southern hybridization. The tTA-encoding transgene was identified by using primers corresponding to the hCMV promoter from −50 to −33 (5′-GGC GTG TAC GGT GGG AGG-3′) and sequences encoding the *tet* repressor (5′-GCA AAA GTG AGT ATG GGT CC-3′). The resulting PCR product was 280 bp in size. The reporter genes were identified with primers corresponding to the hCMV promoter (see above) and the luciferase gene (5′-GCA ATT GTT CCA GGA ACC AGG GCG-3′) or the nuclear localization signal of the β-galactosidase gene (5′-CGG GAT CCC CCA TGC TCC CC-3′). The PCR product for the luciferase gene was 320 bp long and that for the β-galactosidase transgene was 269 bp long. Three types of transgenic mice were generated: mice that carried the hCMV-tTA gene, the luciferase reporter gene, or the β-galactosidase reporter gene containing sequences encoding a nuclear translocation signal.

Administration of Tetracycline. Slow-release tetracycline pellets (Innovative Research of America) were implanted subcutaneously in the shoulder region using a trochar according to the manufacturer's directions. These pellets released 0.7 mg of tetracycline hydrochloride per day. All pellets were kept in place for 7 days before levels of transgene expression were measured. Transgene expression following tetracycline withdrawal was measured 7 days after pellet removal. Tetracycline pellets were given to five females 1–4 days prior to mating. All females became pregnant, and a total of 50 normal pups were delivered. No toxicity from the tetracycline was seen.

Analysis of Luciferase and β-Galactosidase Activities. To analyze luciferase activity, mice were killed by cervical dislocation, and tissue samples were homogenized by using a Polytron in lysis buffer containing 25 mM glycylglycine, 15 mM Mg_2SO_4, 2 mM EDTA, 1 mM dithiothreitol, and 1% Triton X-100. The homogenate was centrifuged for 5 min at 12,000 rpm (14,000 × *g*), and 100 μl of the supernatant was added to 350 μl of assay buffer (25 mM glycylglycine, 15 mM Mg_2SO_4, 5 mM ATP). Luciferase activity was measured using a Berthold Lumat luminometer (Berthold, Germany) after the injection of 100 μl of a 0.05 mM luciferin solution. The protein concentration of the homogenate was determined by using the Bradford assay (Pierce Coomassie protein assay). Luciferase activities were calculated as relative light units (rlu) per mg of total cellular protein.

β-Galactosidase activity was assayed in whole-tissue samples or in frozen sections. To analyze activity in whole-tissue samples, 5-mm cubes of selected tissues were fixed in 2% paraformaldehyde and 0.02% glutaraldehyde in phosphate-buffered saline (PBS) for 1 hr and then rinsed twice in PBS. Staining for β-galactosidase activity was done at 30°C in a solution containing 0.1% 4-chloro-5-bromo-3-indolyl β-D-galactopyranoside, 2 mM $MgCl_2$, 5 mM EGTA, 0.02% Nonidet P-40, 5 mM $K_3Fe(CN)_6$, and 5 mM $K_4Fe(CN)_6 6H_2O$. After staining, the specimens were embedded in paraffin, 10-μm sections were cut and counterstained with eosin or nuclear fast red, and an examination for blue-colored nuclei was conducted. To analyze β-galactosidase activity during embryogenesis, embryos were fixed for 12 hr at 4°C in PBS containing 1% formaldehyde, 0.2% glutaraldehyde, 0.2% Nonidet P-40, and 2.5 mM deoxycholic acid. Embryos were then bisectioned and stained as described above.

RESULTS

Generation of Transgenic Mice. Three types of transgenic mouse lines were generated: reporter mice containing either the luciferase or the β-galactosidase transcription unit and mice carrying the hCMV-tTA gene. The hCMV *IE1* promoter/enhancer was chosen because it is expressed in a broad spectrum of tissues in transgenic mice (13, 14). The reporter genes, encoding either luciferase or the bacterial β-galactosidase, were under the control of P_{hCMV*1}. Whereas the luciferase reporter gene permitted rapid and sensitive analysis of overall transgene expression in selected organs, the β-galactosidase reporter gene enabled us to specifically identify the expressing cell types. A nuclear localization signal in the transgenic β-galactosidase allowed us to easily distinguish it from endogenous cytoplasmic β-galactosidase activity (12). From the six founder animals carrying the hCMV-tTA gene, five (TA1–TA5) were used to establish lines. From the 13 founder mice carrying the luciferase gene, 6 (LU1–LU6) were used to establish lines, and lines were established from the 4 founder animals (G1–G4) carrying the β-galactosidase gene. Double-transgenic mice carrying the hCMV-tTA gene and one of the two reporter genes were generated through cross breeding.

The Luciferase and β-Galactosidase Genes Were Activated by tTA. Basal expression levels of the luciferase gene and the magnitude of induction by tTA were most thoroughly evaluated in the thigh muscle, thymus, and tongue of the trans-

Table 1. Activation of the luciferase gene in tissues of single transgenic mice from line LU5 and in tissues from double-transgenic lines TA1/LU5, TA2/LU5, TA4/LU5, and TA5/LU5

	Luciferase activity, rlu/mg of total cellular protein (no. of animals analyzed)				
Tissue	LU5	TA1/LU5	TA2/LU5	TA4/LU5	TA5/LU5
Thigh					
− Tc	340 ± 160 (19)	10,400 ± 3,100 (4)**	14,800 ± 6,700 (7)*	32,300 ± 17,300 (9)*	53,300 ± 18,600 (4)*
+ Tc	390 ± 150 (7)	410 ± 290 (4)	90 ± 25 (7)	180 ± 110 (14)	1,500 ± 700 (4)
Post-Tc	ND	ND	ND	43,100 ± 6,000 (3)*	ND
Thymus					
− Tc	210 ± 140 (19)	6,000 ± 2,200 (4)**	11,400 ± 6,000 (7)*	5,300 ± 2,800 (9)*	1,800 ± 650 (4)*
+ Tc	230 ± 160 (7)	100 ± 30 (4)	170 ± 110 (7)	200 ± 140 (14)	290 ± 160 (4)
Post-Tc	ND	ND	ND	3,700 ± 1,000 (3)*	ND
Tongue					
− Tc	560 ± 210 (19)	26,000 ± 21,500 (4)**	12,900 ± 6,800 (7)*	27,200 ± 17,000 (9)**	45,700 ± 23,700 (4)*
+ Tc	520 ± 190 (7)	240 ± 140 (4)	180 ± 130 (7)	4,900 ± 3,800 (14)	1,300 ± 490 (4)
Post-Tc	ND	ND	ND	37,700 ± 8,100 (3)*	ND

Luciferase activities were measured in animals that had not been treated with tetracycline (−Tc), in those that had been treated with tetracycline pellets (+Tc), and in those whose tetracycline pellets had been removed 7 days earlier (post-Tc). ND, not determined. *, The difference of activity in the absence and presence of tetracycline (or in the absence of tetracycline and after removal of the tetracycline pellet) yielded a value of $P < 0.01$; **, the difference of activity in the absence and presence of tetracycline yielded a value of $P < 0.05$.

genic reporter line LU5. The basal luciferase activity in these tissues from 19 mice from line LU5 is reported in Table 1. In most mice, basal activity was close to background levels. On occasion, basal luciferase activities of up to several thousand rlu/mg of protein were measured in tongue and thymus. This suggests that there is some heterogeneity of transgene expression within a single integration site. In several mice, basal luciferase activity was analyzed in up to eight additional tissues (Table 2). The ability of the luciferase transgene in line LU5 to respond to activation by tTA was evaluated after breeding this line into the five trans-activator lines. No luciferase activity was measured in any tissue of mice transgenic for the LU5 and the TA3 locus (data not shown). This suggests that the line TA3 does not produce sufficient tTA to activate expression of the luciferase gene. The other four trans-activator lines produced sufficient tTA to activate the luciferase gene (Table 1). Trans-activation was observed in most tissues. The pattern of activity in the different tissues was similar to those reported for an hCMV *IE1*-CAT transgene (13) and an hCMV *IE1*-neo transgene (14). Levels of luciferase activity in the liver were low even in the presence of the tTA. This can be attributed to the low transcriptional activity of the hCMV *IE1* enhancer in liver cells of transgenic mice (13, 14).

Since endogenous DNA sequences in the vicinity of integration sites can exert strong position effects on the expression of transgenes, we compared the extent of induction by a single trans-activator line with three different luciferase-reporter lines. Trans-activator line TA5 was bred into lines LU1, LU5, and LU6, and the magnitude of luciferase activity was measured. The tTA strongly activated the luciferase reporter gene in several tissues of all three lines (Table 2). The pattern of activation was similar in all three combinations. Specifically, expression in tongue, thigh muscle, and skin could be activated at least 100-fold. No activation of the luciferase gene was observed in the liver. The luciferase gene in an additional line did not respond to trans-activation (data not shown), suggesting that the reporter transgene was silent at this integration site.

The β-galactosidase reporter gene allowed us to analyze induction on a single-cell level. The four lines of mice carrying the β-galactosidase transgene were bred into trans-activator lines. No β-galactosidase activity was observed in mice from any of the four lines that contained only the β-galactosidase gene (data not shown). Blue-stained nuclei were observed in the thigh muscle, tongue, and seminal vesicles of mice carrying both a β-galactosidase reporter and a trans-activator gene from several combinations of adult double-transgenic mice. In all combinations tested, not all nuclei were blue, suggesting that only a subset of cells expressed both transgenes (Fig. 1). Blue-stained nuclei were less consistently found in the thymus, heart, kidney, and cerebrum from double-transgenic mice (data not shown). The tissues (tongue and thigh muscle) that demonstrated β-galactosidase activity in nearly all combinations tested were the same tissues that demonstrated high levels of luciferase activity. Activation of the β-galactosidase reporter gene by tTA was also analyzed during embryonic development. While blue staining was restricted to the spinal ganglia in day 11.5 postcoital embryos (data not shown), it was detectable in several embryonic tissues at postcoital day 16.5 (Fig. 2*B*). Particularly strong expression was seen in the nasal region, pituitary, choroid plexus, thymus, and pancreas of whole embryos. However, tissue sections revealed that not all nuclei were blue in these areas (Fig. 2*C* and data not shown). This nonuniform staining pattern was similar to that observed in sections from adult tissues (Fig. 1).

Luciferase Gene Activity Was Abrogated in the Presence of Tetracycline. To inhibit expression from the reporter genes, slow-release tetracycline pellets were implanted into mice transgenic for both the tTA gene and the luciferase reporter gene. Luciferase activities in thigh muscle, thymus, and tongue were measured after 1 week. Basal levels of luciferase activity were found in all double-transgenic mice receiving tetracycline (Table 1). This illustrates that tetracycline inactivated tTA in transgenic mice. Placebo pellets did not reduce luciferase gene activity (data not shown).

DISCUSSION

We have demonstrated that the tetracycline-responsive promoter $P_{hCMV*-1}$ has low basal activity in most tissues of transgenic mice. In double-transgenic mice that synthesize the tetracycline-responsive trans-activator tTA, $P_{hCMV*-1}$ was strongly activated in many tissues. The induction of gene expression was abrogated by the administration of standard therapeutic doses of tetracycline. No toxicity was observed from the exposure to tetracycline. This inducible promoter

Table 2. Activation of the luciferase target gene in transgenic lines LU1, LU5 and LU6, and induction by the tTA transcription factor from line TA5

Tissue	Luciferase activity, rlu/mg of protein		Fold induction	Luciferase activity, rlu/mg of protein		Fold induction	Luciferase activity, rlu/mg of protein		Fold induction
	LU1	LU1/TA5		LU6	LU6/TA5		LU5	LU5/TA5	
Tongue	110	14,000	130	50	116,000	2300	590	45,700	80
Thigh	240	39,500	160	130	7,700	60	350	53,300	150
Liver	20	80	4	10	120	12	20	50	2
Thymus	40	1,400	35	10	8,100	800	220	1,800	8
Heart	50	400	8	110	1,600	15	20	12,300	600
Skin	110	6,700	60	9700	267,000	30	350	73,000	200
Duodenum	15	150	10	2800	21,000	7	10	1,200	120
Colon	80	800	10	170	9,000	55	100	600	6
Whole brain	700	750	1	20	1,600	80	70	500	7
Lung	100	30	0	20	1,500	75	300	300	1
Spleen	20	1	0	350	57,000	160	130	10,700	80
Kidney	10	170	17	10	2,900	290	50	400	8
Seminal vesicle	ND	ND	ND	80	404,000	5000	1000	1,323,000	1300
Testes	ND	ND	ND	1300	2,600	2	360	23,000	64
Uterus	1	470	470	ND	ND	ND	ND	300	ND
Ovary	1	90	90	ND	ND	ND	ND	1,900	ND
Mammary gland	50	1	0	ND	ND	ND	ND	ND	ND

ND, not determined.

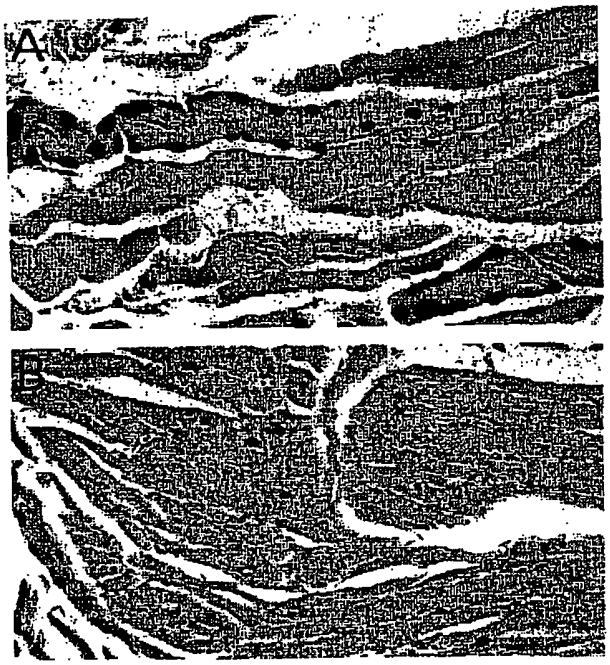

FIG. 1. β-Galactosidase activity in adult tissue sections from thigh muscle (*A*) and tongue (*B*) from an adult mouse carrying the G2 line β-galactosidase and TA1 transgenes. Cells with blue nuclei expressed the β-galactosidase gene.

system can therefore provide temporal control over gene expression in transgenic animals. It has been shown recently that the same system is functional in transgenic tobacco plants (15).

Basal Gene Activity. An essential feature of any inducible system is low promoter activity in the inactive state. Basal transcriptional activity in the *tet* repressor/VP16 system is dependent upon the promoter elements of the target gene. The activity of the $P_{hCMV*-1}$ was examined by using both the luciferase and β-galactosidase reporter genes. No measurable β-galactosidase activity was seen in mice carrying only the β-galactosidase transgene. However, measurable levels of luciferase activity were occasionally observed in certain tissues from some mice containing only the luciferase transgene. The fact that basal expression from the β-galactosidase gene was not seen is probably due to the greater sensitivity of the luciferase assay. The sporadic occurrence of measurable levels of luciferase in some mice indicates that there can be transcriptional activity from the hCMV *IE1* core promoter (16, 17). A core promoter containing only a TATA box may provide lower baseline activity (18). However, this suggestion will have to be tested to determine if such a skeletal promoter can be activated when embedded in chromatin.

Inducibility and Repression of Transgenes. Binding of the tTA transcription factor to the *tet* operator sequences in the promoter of the target gene activates transcription. High activation levels can be achieved even at low concentrations of tTA (9). The fact that tTA is capable of activating transcription of target genes in several independent chromosomal loci shows that the trans-activator can gain access to bacterial control elements packaged into chromatin in differentiated tissues. Trans-activator protein produced by four independent lines of transgenic animals activated luciferase target genes to a similar degree. This may indicate that position effects do not have a dramatic influence on the magnitude of target gene activation. Activity of the luciferase gene in double-transgenic mice was abrogated by administering tetracycline. The therapeutic levels of tetracycline released from the subcutaneously placed pellets were enough to interfere with binding of tTA to the *tet* operator sequences of $P_{hCMV*-1}$. The effect was reversible after removal of the tetracycline pellet. Specific evaluation of the kinetics of repression of gene expression following tetracycline administration or release of repression after tetracycline withdrawal was not made in this study. However, we can state that luciferase activity in double-transgenic mice was fully repressed after 7 days of tetracycline administration. Induction of gene expression followed withdrawal of tetracycline.

Variability and Mosaicism. Expression of the luciferase gene in mice carrying also the hCMV-tTA gene varied between animals from any given line and even between littermates. This variability may be inherent in the hCMV *IE1*

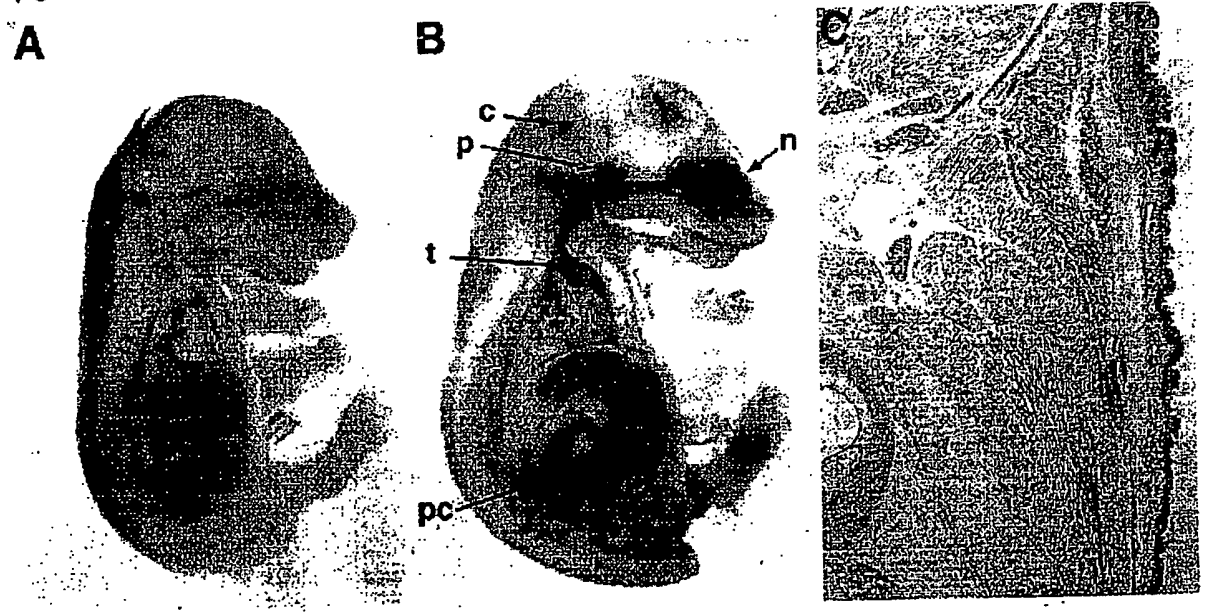

FIG. 2. β-Galactosidase activity in bisectioned day 16.5 postcoital embryos. (*A*) Embryo carrying the G4 line β-galactosidase transgene. (*B*) Embryo carrying the G4 and TA4 transgenes. c, Chloroid plexus; n, nasal region; p, pituitary; pc, pancreas; t, thymus. The blue staining of intestinal tissue is due to endogenous β-galactosidase activity. (*C*) Section from the neck region of the embryo shown in *B*. Cells with blue nuclei expressed the β-galactosidase gene.

promoter/enhancer used to direct expression of the trans-activator. Such variability was also observed in littermates carrying a hCMV *IE1*-CAT transgene (13). Histological analyses for β-galactosidase activity revealed mosaicism. The number of cells expressing the transgene was only a subset of the cells expected to stain in that site, a pattern reminiscent of position-effect variegation (19). Although a possibility, mosaicism cannot be attributed simply to the use of the β-galactosidase reporter gene as this gene has been expressed homogeneously in embryonic, fetal, and adult tissues (20). Mosaicism has been observed with other transgenes (12, 14), and even some endogenous genes (21), and is referred to as incomplete penetrance (12). This stochastic pattern of gene expression may reflect the activity of certain endogenous genes (21) and also may be the cause of the variable penetrance of defects observed in null mutant mice (22, 23).

Other Inducible Systems. Other inducible promoter systems have not offered the degree of control presented by this strategy. Similar to the system reported here, two previously described binary systems consist of a silent target gene that is induced by constitutive or regulated trans-activators (2, 3). In the third reported system, a silent target gene is activated by a site-specific recombinase (4). However, in contrast to the system described here, transgene activity in these binary systems is regulated by the transcription pattern inherent to the promoter controlling the trans-activator gene. No additional manipulation is possible.

A system that uses the *tet* repressor to inhibit gene transcription is another approach to controlling gene activity. In these systems *tet* repressor molecules bind to *tet* operator sequences located at the transcriptional start site and block gene transcription in the absence of tetracycline. In the presence of tetracycline binding of the tet repressor to the *tet* operator is greatly reduced and transcription is activated (24). Repression of transcriptional activity to basal levels has been achieved in the presence of ≈500,000 tet repressor molecules per cell. Such a concentration can only be obtained with strong promoters, such as the 35S promoter from the cauliflower mosaic virus (24). Therefore, it may be difficult to achieve a repression of the transgene with housekeeping or standard tissue-specific promoters.

The induction of transgenes through the withdrawal of tetracycline can have specific advantages for some experiments. For example, when analyzing the roles of oncogenes, growth factors, or tumor suppressor genes on tumor formation, a long period of gene activation may be required (25–28). If a tetracycline-responsive promoter is used to control oncogene expression, it may be convenient to have the animals off tetracycline during this time.

In conclusion, the tetracycline regulatory system can provide temporal control of transgene expression. It should be useful for experiments designed to address certain biological questions in transgenic animals. For example, temporal control of the induction of growth modulators, oncoproteins, and other proteins participating in developmental processes could provide further definition to their roles in normal growth and tumorigenesis. The effects of expressing potentially deleterious genes can be studied, since these genes can be rendered inactive by using tetracycline. Alternatively, the system can be combined with one of the site-specific recombinases (4) and used to delete genes at specific time points during development.

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